G2201-C, A NEW CYCLOPENTENEDIONE ANTIBIOTIC, ISOLATED FROM THE FERMENTATION BROTH OF *STREPTOMYCES CATTLEYA*

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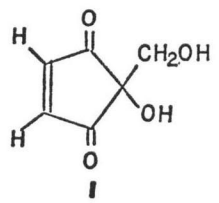
Streptomyces cattleya produced a new cyclopentenedione antibiotic, G2201-C [$C_6H_6O_4$ (I)], which is moderately active *in vitro* against Gram-positive bacteria, weakly active against Gram-negative bacteria, and inactive against fungi. G2201-C is toxic to mice.

An antibiotic, called G2201-C, was isolated from the culture broth of *Streptomyces cattleya*, an organism that has been reported to produce the β-lactam antibiotic, thienamycin.[1] Physical and chemical measurements of crystalline G2201-C suggest it to be 2-hydroxy-2-hydroxymethylcyclopent-4-ene, 1,3 dione (I).

This paper describes the production, isolation and properties of this antibiotic.



I

Experimental

Antibiotic detection and assay

Antibiotic production and isolation were followed by t.l.c. Samples of broth or of extracts of broth were applied by means of micropipettes (Drummond Scientific Co., Broomall, Pennsylvania, U.S.A.) to thin-layers of cellulose (20×20 cm; 13254 containing fluorescent indicator; Eastman Kodak Co., Rochester, N.Y., U.S.A.). The thin-layer plates were developed with acetonitrile - water (7 : 3 by volume) at 24°C, air-dried and examined under U.V. light (254 and 356 nm). To detect active material the plates were sprayed with a solution of agar (0.7%, no. 3, Oxoid Ltd., London, England) then overlayered with nutrient agar (beef extract, L29, Oxoid Ltd., 0.57%; bacteriological peptone, L34, Oxoid Ltd., 0.71%; sodium chloride, 0.36%; and agar (no. 3, Oxoid Ltd.), 0.86%) containing test organism and 2,3,5-triphenyl tetrazolium chloride, 0.006%. Test organisms were *Staphylococcus aureus* Oxford H strain V1, *Escherichia coli* NCIB 9482 and a *Flavobacterium* species (Glaxo strain C2116). The overlayered plates were incubated at 37°C for 16 hours and the Rf values recorded.

The minimum inhibitory concentration (MIC) of the antibiotic was determined for various bacteria and fungi by serial dilution assays in tubes as described by NOBLE *et al.*[2]

Fermentation

Streptomyces cattleya, NRRL 8057, was grown for 14 days at 28°C on slopes containing yeast extract (L21, Oxoid Ltd.), 1%; glucose, 1%; $MgSO_4 \cdot 7H_2O$, 0.005%; KH_2PO_4, 0.018%; Na_2HPO_4, 0.019% and agar (no. 3, Oxoid Ltd.), 2%, made up in distilled water and adjusted to pH 6.5.

Growth from one slope was suspended in 10 ml sterile distilled water and 1 ml of the suspension was transferred to 50 ml of sterilised medium at pH 7 containing soya bean meal (J. Bibby & Sons Ltd., Liverpool, Lancashire, England), 0.5%; yeast extract (L21, Oxoid Ltd.) 0.5%; tryptone (L42,

Oxoid Ltd.), 0.5%; glycerol (Evans Medical Ltd., Liverpool, Lancashire, England), 1% and K_2HPO_4, 0.02%, made up in tap water. The inoculated medium was shaken (220 rev/min on a rotary shaker with a 2-inch throw) in a 250-ml baffled conical flask for 48 hours at 28°C.

Portions (1.5 ml) of the fermentation described above were transferred to 75 ml amounts of sterilised medium at pH 7.2 containing peptone (L34, Oxoid Ltd.), 2%; Lab-Lemco beef extract (L29, Oxoid Ltd.), 0.8%; glucose, 1.5%; glycerol (Evans Medical Ltd.), 1% and $CaCO_3$, 0.04%, made up in distilled water. The inoculated medium was shaken as already described for 48 hours at 25°C.

Antibiotic isolation

Broth (4 litres) at harvest was adjusted to pH 3.5 with formic acid and centrifuged (Mistral 6L Centrifuge, Measuring and Scientific Equipment Ltd., London, England; 2,000 rev/min, 30 minutes, 4°C) to sediment mycelium. The supernatant liquid (3.5 litres) was divided into halves and each was applied to a column (30 × 4 cm) containing 350 ml charcoal (granular activated carbon, type CAL; Calgon Corporation, Pittsburgh, Pennsylvania, U.S.A.). Before use the charcoal was washed with hot 1 N HCl, acetone then water. After the supernatant liquid had percolated into the charcoal each column was washed with 500 ml water and active material was eluted with 1 litre acetone - water (1: 1 by volume). The eluate was evaporated to remove acetone and the aqueous solution remaining was freeze-dried.

Each freeze-dried solid was extracted twice with 250 ml methanol and the combined extracts were filtered to remove inactive solid. The filtered extract was evaporated to 70 ml and applied to a column of Sephadex LH20 (140 × 5 cm; Pharmacia Fine Chemicals AB, Uppsala, Sweden) packed in *n*-butanol - methanol - water (4: 1: 2 by volume). Elution was with the same solvent.

Active fractions were evaporated to dryness in the presence of silica (20 ml Woelm 04526 for dry column chromatography; ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.). The resultant solid was applied to a column of the same silica (50 × 2.5 cm) packed dry. Elution was with distilled ethyl acetate. Active fractions were combined and the chromatography was repeated as described except that the bed size was 82 × 2 cm.

Active fractions were combined, evaporated to 2 ml and applied to a column of Sephadex LH20 (70 × 2 cm) packed in ethyl acetate - methanol (3: 1 by volume). Elution was with the same solvent. Active fractions were combined and evaporated. The residue was kept under reduced pressure for 18 hours at −20°C. Yellow crystals (400 mg) were collected and recrystallised from diethylether (40 ml at 25°C) to yield 176 mg yellow crystals.

Thin-layer chromatography

Samples of G2201-C (20 μg) were applied to thin-layers of cellulose containing fluorescent indicator (20 × 20 cm; 13254; Eastman-Kodak Co.) and to thin-layers of silica containing fluorescent indicator (20 × 20 cm; $60F_{254}$, 5735; E. Merck, Darmstadt, Germany). Development was with the solvents given in Table 1 at 24°C. After development thin-layer plates were dried in a stream of cold air and examined for the presence of antibiotic. The antibiotic was detected by overlayering the plate with nutrient agar containing a test organism and recording zones of inhibition of growth of the organism as described above. G2201-C was also detected by a strong quenching of fluorescent light if the plate was exposed to ammonia vapour (sp.gr. 0.88) in a closed chamber for 2 minutes then examined under U.V. light (254 or 356 nm) or by a blue colour after spraying the plate with 0.5% blue tetrazolium (BDH Chemicals Ltd., Poole, Dorset, England) in methanol - 2 N NaOH (1: 10 by volume).

Spectroscopy

The mass spectrum (electron ionisation and field desorption) was recorded on a Varian MAT 311A spectrometer and the carbon-13 nmr spectrum on a Bruker HX90E spectrometer.

Evaporation

Evaporation of solvent was under reduced pressure with a rotary evaporator.

Solvents

All solvents were of analytical grade or were redistilled.

Acute mouse toxicity

Test compound was dissolved in isotonic saline and dilutions made with the same solvent. Each concentration of compound was administered i.p. to a group of 5 albino female mice (Charles River, Harefield strain). Each mouse weighed 20 g and received 0.2 ml of the appropriate test solution. Survivors were counted daily for five days.

Results

Physical and Chemical Properties

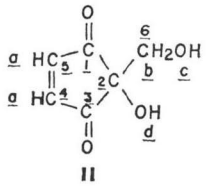
G2201-C is a yellow crystalline antibiotic, soluble in water, methanol and acetone and slightly soluble in chloroform and diethyl ether. In solution at pH values greater than 7 the yellow colour intensifies and antibiotic activity is lost.

Crystals of G2201-C melt at 53°C. A field desorption mass spectrum indicated a molecular ion at *m/e* 142; this ion was shown by electron ionisation mass spectrometry to have an accurate mass of 142.0195, corresponding with a molecular formula of $C_6H_6O_4$. Elementary analysis gave C, 50.30; H, 4.21. $C_6H_6O_4$ requires C, 50.73; H, 4.22%.

Maxima (nm with $E^{1\%}_{1cm}$ values within brackets) were obtained for a 0.001% aqueous solution of G2201-C at 220 (840) and 225 (780). In 0.05 N NaOH a transient maximum at 290~310 nm and in 0.05 N NH_4OH at 275~282 nm was noted.

The infrared spectrum in Nujol had absorptions at 3460~3220 (hydroxyl) and at 1760, 1710 and 1565 cm^{-1} (CO–C=C–CO).

A 100 MHz proton nmr spectrum of a solution of G2201-C in deuterated acetone had peaks [assignments (**II**) given as underlined letters] at τ 2.54 (s; 2H, *a*), 4.95 (s; 1H, *d*), 5.82 [t (6 Hz); 1H, *c*] and 6.26 [d (6 Hz); 2H, *b*]. An off-resonance decoupled 22.5 MHz carbon-13 nmr spectrum of a solution of G2201-C in deuterated dimethyl sulphoxide showed signals [assignments (**II**) given as underlined numerals] at δ 205.2 (s; 1 and 3), 149.9 (t; 4 and 5),* 74.0 (s; 2) and 62.9 (*t*; 6).



II

Rf values for G2201-C obtained after thin-layer chromatography on cellulose and on silica are given in Table 1.

Biological Properties

The antibacterial spectrum is given in Table 2. G2201-C is moderately active against a range of Gram-positive bacteria but is not very active against most of the strains of Gram-negative bacteria tested.

Minimum inhibitory concentrations of greater than 250 μg/ml were obtained against strains of *Candida albicans, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Neurospora sitophila, Fusarium oxysporum, Trichophyton mentagrophytes* and *Microsporium canis.*

The LD_{50} (mice i.p.) was about 19 mg/kg body weight.

Discussion

Streptomyces cattleya produces the β-lactam antibiotic, thienamycin.[1] The organism, when grown in shake culture on a medium containing peptone and beef extract nitrogen sources and

* The triplet structure of the signal at δ 149.9 is in accordance with molecular symmetry and arises from virtual coupling effects between one ^{13}C nucleus at 4 or 5 and the approximately isochronous protons attached to C_4 and C_5.

Table 1. T.l.c. of G2201-C

Solvent	Support	Rf
Benzene	Silica	0
Chloroform		0
Ethyl acetate		0.50
Dioxane		0.80
Acetone		0.78
Methanol		0.78
Butan-1-ol - acetic acid - water (3: 1: 1)		0.48
Ethyl acetate - methanol (3: 1)		0.76
Propan-1-ol - pyridine - acetic acid - water (15: 10: 3: 12)	Cellulose	0.95
Butan-1-ol - acetic acid - water (3: 1: 1)		0.67
Butan-1-ol - methanol - water (4: 1: 2)		0.70
Acetonitrile - water (7: 3)		0.85

Table 2. Antibacterial spectrum of G2201-C

Organism*	MIC (μg/ml)
Staphylococcus aureus 663	31
Staphylococcus aureus 853E	31
Micrococcus sp. 1810E	31
Streptococcus faecalis 850E	62
Streptococcus pneumoniae 1910E	8
Bacillus cereus NCIB 8849	16
Escherichia coli 1193E	>250
Escherichia coli 1507E	>250
Escherichia coli 1852E	31
Escherichia coli C1343	125
Klebsiella aerogenes 1082E	>250
Klebsiella aerogenes 1522E	>250
Enterobacter cloacae 1051E	>250
Enterobacter cloacae 1321E	>250
Proteus morganii 235	>250
Proteus mirabilis 431E	31
Pseudomonas aeruginosa 1371E	125
Serratia marcescens 1324E	>250

* All organisms are Glaxo strains except the *Bacillus* species.

glucose and glycerol also produced a less polar antibiotic (G2201-C) active against *Staphylococcus aureus* and some strains of *Escherichia coli*.

G2201-C was isolated from culture broths by adsorption on charcoal and after elution was further purified by chromatography on Sephadex LH20 with butanol - methanol - water and with ethyl acetate - methanol and on silica with ethyl acetate.

G2201-C, a yellow crystalline polar compound, is moderately active against Gram-positive organisms, weakly active against some Gram-negative organisms but is not active against fungi. It is toxic to mice.

G2201-C is a cyclopentenedione antibiotic closely related to pentenomycins I and II produced by *Streptomyces eurythermus*[3]. The pentenomycins were also moderately active against Gram-positive and Gram-negative bacteria but were apparently much less toxic to mice than G2201-C.

Acknowledgement

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